Autoradiographic Studies of Human Lymphocytes Cultured *in Vivo** (33633)

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Cell culture studies with phytohemagglutinin (PHA) have clearly established the small lymphocyte as a cell capable of reverting from a quiescent state to a blast form that synthesizes DNA and undergoes division. The relationship of this *in vitro* proliferation to *in vivo* function, remains to be elucidated. However, by its resmblance to the behavior of lymphocytes stimulated immunologically *in vivo* (1), the *in vitro* response to PHA may represent a model of the cell's immunologic competence. The present paper reports on the proliferation of human lymphocytes cultured in diffusion chambers placed into the peritoneal cavities of rats. This system has yielded consistently good results when applied to rat lymphocytes which are difficult to maintain *in vitro* (2–5). The diffusion chamber technique may thus represent an improvement in the method of culturing lymphocytes, as it more closely approximates the true *in vivo* environment.

Materials and Methods. The technique for culturing blood lymphocytes in diffusion chambers was previously described (2). Heparinized blood (100 units/ml) obtained from a healthy donor, was centrifuged at 65*g* for 8 min and the leukocyte-rich supernatant was removed. Phytohemagglutinin-P (Difco PHA-P, reconstituted in 30 ml of medium 199) was added to one half of the leukocyte suspension at a concentration of 0.2 ml/ 6×10^6 cells. The remaining half of the cells, suspended without PHA, served as a control. Both preparations were placed in an ice bath for 45 min, then centrifuged at 163*g* for 10 min. The sedimented button of leukocytes obtained was suspended in medium 199 to a final cell concentration of 30–40 $\times$ 10^6 cells/ml and then introduced into Millipore diffusion chambers (0.2-ml capacity, 0.45-μ diam pore size). Three to four chambers were implanted into the peritoneal cavity of each adult rat. Studies of these cultures for morphologic changes and DNA synthesis, as judged by tritiated thymidine (T-^{3}H) incorporation, were made at daily intervals up to 6 days. Pulse labeling with T-^{3}H (sp act. 6iCi/mmole, Schwarz BioResearch) was performed *in vivo* by injecting the isotope into the peritoneal cavity of each chamber-bearing host (0.5 μCi/g, in 1 ml) 1 hr before sacrifice. Differential counts and labeling indices were determined by enumerating 1000–2000 cells from each culture.

For cell cycle analysis, the cultures were grown in host rats for 48–50 hr, at which time all host animals were given a single intraperitoneal injection of T-^{3}H and then were sacrificed serially. The cultures were harvested at 10, 20, 30, 60, and 120 min, and thereafter at 2–4-hr intervals up to 26 hr after administering the pulse label. The smears prepared from the chamber contents were processed by autoradiography (Kodak Nuclear Emulsion NTB-2; 5-weeks exposure at 4°; developed in Kodak D-19), and the subcycle durations were estimated using the labeled mitoses technique of Wimber (6).

Results. a. Blast transformation. Human lymphocytes stimulated with phytohemagglutinin (PHA) in diffusion chambers, exhibited the morphologic transformation, time se-

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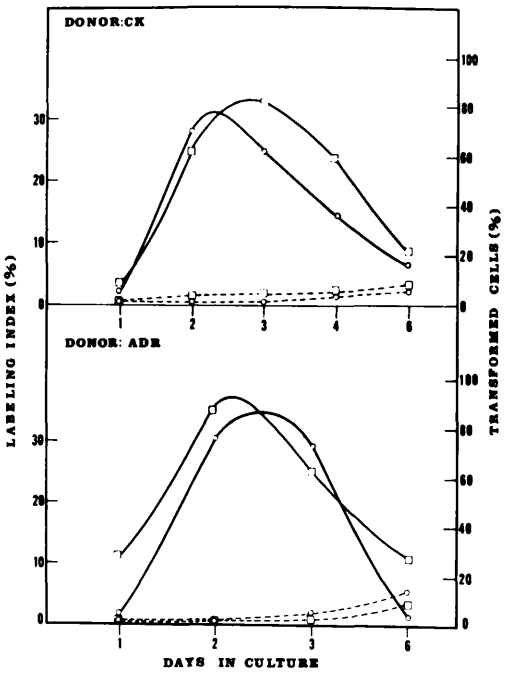

FIG. 1. Labeling indices (LI) and percentage of transformed cells (transitionals and blasts) for human lymphocytes cultured *in vivo*. (O—), LI, PHA-treated cultures; (O - -), LI, control cultures; (□ —), percentage of transformation, PHA-treated cultures; and (□ - -), percentage transformation, control cultures.

quence of blast development, and initiation of DNA synthesis similar to that observed *in vitro* (7–9). The peak response in the percentage of blast cells generally occurred at 2–3 days (Fig. 1). Control cultures showed none or only a minimal number of blast cells (2–3%) which developed at the later culture periods. In addition, these cultures contained a heterogeneous group of enlarged cells resembling macrophages, reticulum cells, and fibroblasts which comprised 4–7% of the population (not included among blastoid cells in Fig. 1). Less than 1% of these non blastic elements were observed in the PHA-treated cultures. Thus, within the time interval studied, the lymphocyte response in PHA-stimulated cultures was characterized by the presence of a very large percentage of blasts whose presence was directly attributed to the effect of the mitogenic agent.

b. DNA synthesis. Figure 1 shows the pattern of DNA synthesis for human lymphocytes cultures *in vivo*. Coinciding with the morphologic changes, the peak in DNA synthesis generally occurred at 2–3 days after exposure to PHA. The differential labeling indices revealed that the transformed cells accounted for 92–99% of the labeled population. At the later culture periods (4–6 days), control cultures exhibited a slight increase in the labeling index which was due mainly to the heterogenous group of enlarged cells.

C. Cell cycle analysis. Previous studies showed that following a single interperitoneal injection of T-^{3}H to host rats, the label was available to rat cells within the diffusion chambers, for only 30–60 min (10). Thus, a pulse label was achieved. When human lymphocyte cultures were similarly pulse labeled, the first labeled metaphases were observed at 2 hr after exposure to T-^{3}H, indicating a minimum T_{G2} [3] of about 2 hrs (Fig. 2). A maximum of 82–84% labeled mitoses was observed between 6 and 10 hours; thereafter, this percentage declined. Thus, $T_{G2} + \frac{1}{2} T_M$ was estimated at about 3.25 hr and minimum T_S at 9–10 hr.

If it is assumed that the cells in these cultures were dividing asynchronously (as suggested from Table I mitotic indices) under steady state conditions, the labeling index (LI in % = Ns/Nc × 100)[4] can be used to estimate the cell cycle time, since LI $\cong$ T_s/T_C. Using a T_S of 9–10 hr, and the average of the labeling indices found for blast cells (about 55%) at 30, 60, and 120 min (by min only few labeled cells have entered mitosis), an average cell cycle time of 16.3–18.1 hr was estimated.

In the interval following the pulse label with T-^{3}H, a marked increase was noted in the percentage of small cells labeled. These cells displayed morphologic features that were similar to those of small lymphocytes. From a labeling index of 0.8% at 1 hr, the

[3] T_{G1} = postmitotic presynthetic period. T_S = DNA synthesis time. T_{G2} = post-DNA synthesis period; T_M = mitotic time; T_C = cell cycle time.

[4] Ns = number of cells in DNA synthesis; Nc = number of cells in the population.

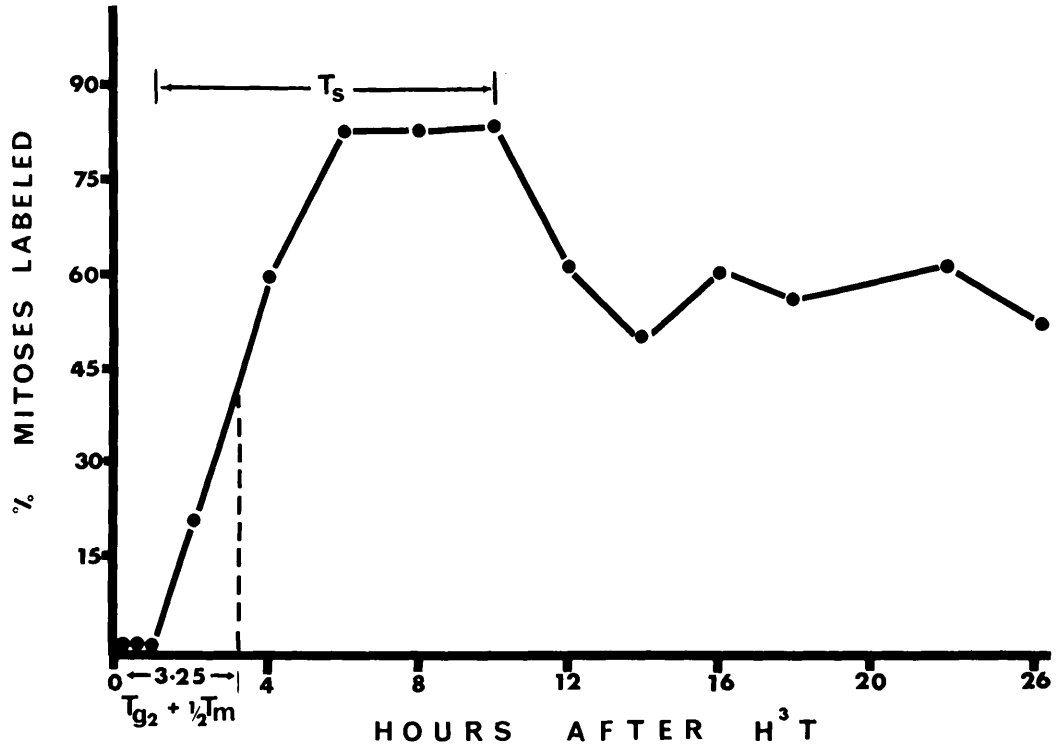

FIG. 2. Change in the percentage of labeled mitoses with time following the pulse labeling of 48–50-hr old cultures with T-^{3}H: T^3 = minimum time for DNA synthesis; mitotic figures scored totaled 1059.

value increased to 16.3%, 26 hr after exposure to T-^{3}H (Table I). These labeled small cells most likely represented the progeny of labeled blast cells.

Discussion. The enlargement and proliferation of human lymphocytes cultured in diffusion chambers followed much the same pattern as that observed *in vitro* (7–9, 11). However, the peak in the percentage of cells synthesizing DNA was achieved somewhat earlier, as the *in vivo* system required 2–3 days, compared with 3–4 days *in vitro* (12, 13). A small number of blasts and other large mononuclear cells appeared in some control cultures during the later culture periods. Since the original inoculum was not a purified lymphocyte suspension, the origin of these enlarged cells is obscure. Spontaneous transformation in unstimulated lymphocytes has been reported for *in vitro* cultures (14). Whether immunologic reactions associated with the *in vivo* culture of heterologous cells may also account for the presence of these enlarged cells, is not known. However, PHA-treated and control cultures exhibit sufficiently marked differences which clearly distinguish the two patterns of response.

Good cellular morphology and a high level of mitotic activity which can be obtained for extended periods, make the diffusion chamber culture system ideally suited to the type of cell cycle analysis applied in the present study. The subcycle values estimated for these cultures using the labeled mitoses method, tend, on the whole, to be shorter in duration than the values reported for *in vitro* cultures. Thus, the minimum T_{G2} reported was 3.5–6 hr (15, 16) compared with 2 hr in the *in vivo* system. The rough estimate of T_c (16–18 hr) was also in contrast to the 22–38.5 hr observed *in vitro* (16–18). Our value may represent a slight overestimate if the PHA blast population increased in a non-steady state manner with time. The DNA synthesis time was in close agreement with the value obtained for human lymphocytes

TABLE I. Labeling (LI) and Mitotic Indices (%) for PHA-treated Cultures Pulse Labeled with T-^{3}H and Reaped Serially for Cell Cycle Studies. The cultures were pulse labeled after 48 hr of incubation with PHA.

Time	Mitotic index	LI blasts	LI transitionals	LI lymphocytes	LI population
(min)					
10	1.25	26.0	10.0	0.2	11.4
30	0.65	61.1	41.9	7.4	19.7
(hr)					
1	0.60	35.5	24.6	0.8	14.7
2	1.00	68.0	53.9	9.3	31.9
4	0.50	45.6	32.2	3.4	11.3
6	0.57	51.3	54.7	11.1	26.7
8	1.00	43.5	18.8	8.5	19.5
10	0.65	70.2	49.9	7.3	37.8
12	0.85	44.2	37.4	12.5	29.2
14	1.30	36.0	26.8	8.6	23.0
16	1.50	49.3	50.0	26.4	40.8
18	0.75	45.8	41.8	18.8	33.5
22	0.80	57.7	41.7	21.9	36.6
26	0.70	46.0	40.0	16.3	38.5

grown *in vitro* (19). It is possible that within the *in vivo* milieu, pH regulation and metabolic exchange may be enhanced by the host's own homeostatic mechanisms, thus promoting optimal conditions for cell growth and multiplication.

In this study, the entry rate of labeled cells into mitosis (Fig. 2, indicated by the ascending limb of the curve), was very gradual, due perhaps to variations in T_{G2}. In addition, labeled mitoses never exceeded 84%. The reason for this is not clear, but could be attributed to factors such as a heterogeneous cell population with respect to proliferative rates, extended intracellular storage of water soluble DNA precursors preceding DNA synthesis, or the inability to label all the cells that were in DNA synthesis. Inaccessibility of label to some cells may be ruled out since, (a) all cultures were consistently below the 100% level of labeled mitoses, and (b) in similar studies with rat lymphocytes, 100% labeled mitoses was achieved using the same technique of pulse labeling cells *in vivo* (10). This inability to achieve 100% labeled mitoses has also been noted *in vivo* for human hematopoietic precursor cells (20).

Serial differential counts of cultures pulse labeled with T-^{3}H revealed a progressive increase with time in the labeling of small mononuclear cells (Table I). These cells possessed the morphologic features of the typical small lymphocyte, namely, pachychromatic nucleus and scant cytoplasm. Barring such factors as tritium reutilization or labeled precursor storage, it might be concluded that the small labeled cells arose as progeny of the transformed cells. The appearance of small ^{3}H-labeled lymphocyte-like cells has also been observed in cultures of rat lymphocytes treated with PHA (21, 22). Using Iododeoxyuridine-^{125}I (a thymidine analogue with minimal reutilization) to monitor T-^{3}H reutilization, small labeled cells were also observed with time after the cultures were pulse labeled with the isotope (21). It was concluded that the blast cells reverted to this quiescent small lymphocyte stage following their intense proliferative phase, perhaps after the stimulatory effect of the mitogen was dissipated. Thus, these studies confirm the similarity of the morphologic and metabolic reactions of lymphocytes in cell culture to the immunoproliferative reaction observed *in vivo* (1). The small lymphocyte [the central cell of the immunocyte complex, (23)] transforms into a blast cell (immunoblast) which

undergoes proliferation, giving rise to a new population of lymphocytes.

Summary. Human lymphocytes were cultured in diffusion chambers implanted into the peritoneal cavities of rats. In cultures treated with PHA, the pattern of morphologic transformation and initiation of DNA synthesis paralleled closely similar studies made *in vitro*. Immunologic stimulation of the human cells by the heterologous host was presumably minimal or absent since control cultures (without PHA) showed no significant degree of blastogenesis when compared with PHA-treated cultures. Minimum DNA synthesis time determined for the PHA-stimulated lymphocytes was 9–10 hr. Values found for the duration of T_c (16–18 hr) and minimum T_{G2} (2 hr) were shorter than those reported for similar studies *in vitro*. The *in vivo* culture method using Millipore chambers appears to offer a more physiologic method for studying lymphocytes.

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